

## Amendments to the Specification

Please amend the paragraph beginning on page 1, line 5 of the instant specification as follows.

### Related Applications

This application claims the benefit of priority is a continuation-in-part of ES200302587, filed on November 5, 2003, and which is a continuation-in-part of ES200300252, filed January 31, 2003, each of which is incorporated herein by reference in its entirety.

Please amend the title of the instant specification as follows.

Immune Regulation Based on the Targeting of Early Activation Molecules Depletion of CD69+  
Cells

Please amend the paragraph beginning at page 12, line 26 of the instant specification as follows.

In a preferred embodiment, the method includes administering an early activation molecule antagonist in combination with a second agent, e.g., with one or more therapeutic agents, e.g., a therapeutic agent or agent for treating unwanted cell proliferation. The second agent can be an antibody or a non-antibody agent. Therapeutic agents include, for example, one or more of a chemotherapeutic agent, a radioisotope, and a cytotoxin. Examples of chemotherapeutic agents include ~~taxol~~ TAXOL®, cytochalasin B, gramicidin D, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicine, busulfan, cisplatin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, chlorambucil, gemcitabine, actinomycin, procaine, tetracaine, lidocaine, propranolol, puromycin, maytansinoids and analogs or homologs thereof. Additional therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, CC-1065, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine, vinblastine, ~~taxol~~ TAXOL® and

maytansinoids). Radioisotopes can include alpha, beta and/or gamma emitters. Examples of radioisotopes include  $^{212}\text{Bi}$ ,  $^{213}\text{Bi}$ ,  $^{131}\text{I}$ ,  $^{211}\text{At}$ ,  $^{186}\text{Re}$ ,  $^{90}\text{Y}$  and  $^{117}\text{Lu}$ . The early activation molecule antagonist can be administered prior to, concurrent with or after administration of the therapeutic agents.

Please amend the paragraph beginning on page 16, line 28 of the instant specification as follows.

In a preferred embodiment, the method includes administering an early activation molecule depletor in combination with a second agent, e.g., with one or more therapeutic agents, e.g., a therapeutic agent or agent for treating cancer. The second agent can be an antibody or a non-antibody agent. Therapeutic agents include, for example, one or more of a chemotherapeutic agent, a radioisotope, and a cytotoxin. Examples of chemotherapeutic agents include ~~taxol~~ TAXOL®, cytochalasin B, gramicidin D, mitomycin, etoposide, teniposide, vincristine, vinblastine, colchicin, busulfan, cisplatin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, chlorambucil, gemcitabine, actinomycin, procaine, tetracaine, lidocaine, propranolol, puromycin, maytansinoids and analogs or homologs thereof. Additional therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, CC-1065, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine, vinblastine, ~~taxol~~ TAXOL® and maytansinoids). Radioisotopes can include alpha, beta and/or gamma emitters. Examples of radioisotopes include  $^{212}\text{Bi}$ ,  $^{213}\text{Bi}$ ,  $^{131}\text{I}$ ,  $^{211}\text{At}$ ,  $^{186}\text{Re}$ ,  $^{90}\text{Y}$  and  $^{117}\text{Lu}$ . The early activation molecule depletor can be administered prior to, concurrent with or after administration of the therapeutic agents.

Please amend the paragraph beginning on page 64, line 30 of the instant specification as follows.

Similarly, in the HuMAB mouseHUMAB MOUSE®, available from Medarex and GenMab, mouse genes for creating antibodies are inactivated and replaced with many of the key

gene sequences from unarranged human antibody genes coding for heavy and light chains. Another mouse available is the XeneMouseXENOMOUSE® from Abgenix which has approximately 80% of the human heavy chain antibody genes and a significant amount of the human light chain genes. Other transgenic mice are available for generating human antibodies which contain complete sets of the variable and constant genes found in the corresponding natural human immunoglobulin loci. These mice, also referred to as Kirin TC-mice TC MICE™ are available from Medarex. The Kirin TC-mice TC MICE™ are "transchromosomal," meaning that the mouse genes for creating antibodies have been inactivated and have been replaced by the human chromosomes containing all of the human antibody genes, including all heavy chain classes that encode all isotypes (IgG1-4, IgA1-2, IgD, IgM and IgE). Also available from Medarex, is the KM-mouseKM MOUSE®, a crossbred mouse that combines the characteristics of the HuMAB Mouse HUMAB MOUSE® with Kirin's TC Mouse MOUSE™. The KM-mouse MOUSE®, like the Kirin TC Mouse MOUSE™, retains the capability to produce all human antibody isotypes. Splenocytes from these transgenic mice immunized with the antigen of interest are used to produce hybridomas that secrete human mAbs with specific affinities for epitopes from a human protein (see, e.g., Wood et al. International Application WO 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg et al. International Application WO 92/03918; Kay et al. International Application 92/03917; Lonberg, N. et al. 1994 *Nature* 368:856-859; Green, L.L. et al. 1994 *Nature Genet.* 7:13-21; Morrison, S.L. et al. 1994 *Proc. Natl. Acad. Sci. USA* 81:6851-6855; Bruggeman et al. 1993 *Year Immunol* 7:33-40; Tuallion et al. 1993 *PNAS* 90:3720-3724; Bruggeman et al. 1991 *Eur J Immunol* 21:1323-1326). The procedures for the isolation of human mAbs are identical to those used for conventional mouse immunization and hybridoma production (reviewed in Brüggemann and Taussig, 1997; Brüggemann and Neuberger, 1996; Jakobovits, 1995).

Please amend the paragraph beginning on page 69, line 27 of the instant specification as follows.

A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include taxol TAXOL®, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin, maytansinoids, e.g.,

maytansinol (see US Patent No. 5,208,020), CC-1065 (see US Patent Nos. 5,475,092, 5,585,499, 5,846,545) and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, CC-1065, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine, vinblastine, ~~taxol~~ TAXOL® and maytansinoids).

Please amend the paragraph beginning on page 95, line 18 of the instant specification as follows.

The early activation molecule antagonist can be administered in combination with one or more therapeutic agents, *e.g.*, a therapeutic agent or agent for treating or preventing unwanted cell proliferation. The therapeutic agents include, for example, one or more of a chemotherapeutic agent, a radioisotope, and a cytotoxin. Examples of chemotherapeutic agents include ~~taxol~~ TAXOL®, cytochalasin B, gramicidin D, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, busulfan, cisplatin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, chlorambucil, gemcitabine, actinomycin, procaine, tetracaine, lidocaine, propranolol, puromycin, maytansinoids and analogs or homologs thereof. Additional therapeutic agents include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, CC-1065, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine, vinblastine, ~~taxol~~ TAXOL® and maytansinoids). Radioisotopes can include alpha, beta and/or gamma emitters. Examples of radioisotopes include <sup>212</sup>Bi, <sup>213</sup>Bi, <sup>131</sup>I, <sup>211</sup>At, <sup>186</sup>Re, <sup>90</sup>Y and <sup>117</sup>Lu. The early activation molecule antagonist and the therapeutic agents can be administered simultaneously or sequentially. In addition, the early activation molecule antagonists can be

administered in conjunction with other cancer treatment modalities such as a surgery to remove all of part of the cancerous tissue or the organ containing the cancerous tissue.

Please amend the paragraph beginning on page 115, line 14 of the instant specification as follows.

**RNase protection assay and quantitative real-time RT-PCR analyses.** Joints were homogenized with a Polytron POLYTRON® (Kinematica, Littau, Switzerland) and total RNA was isolated using the Ultraspec ULTRASPEC™ RNA reagent (Biotecx, Houston, Texas). In tumor challenge experiments total RNA was extracted from unfractionated peritoneal cells. RNase protection assays were performed on 2.5 to 5 µg of RNA using the Riboprotect RIBOQUANT™ MultiProbe RNase Protection Assay System (PharMingen, San Diego, California). For RT-PCR, 2 µg of DNaseI-treated RNA were reverse transcribed with MuLV RT (Roche Diagnostics Ltd, Lewes, UK). Real-time PCR was performed in a Lightcycler LIGHTCYCLER® rapid thermal cycler system (Roche) using primers from different exons that generated products of about 200 bp length.

Please amend the paragraph beginning on page 116, line 10 of the instant specification as follows.

**Determination of cytokine production in CIA.** To determine the levels of cytokines in washouts of joint tissue, patellae with adjacent synovium were obtained at mice sacrifice (day 50) in a standardized manner from knee joints as previously described (Lubberts et al., 2000), and incubated in RPMI-1640 medium (200 µl/patella) with 0.1% BSA for 1h at RT. Then, active and total TGF-β1 (EMax EMAX® ImmunoAssay System; Promega Corp., Madison, Wisconsin), and IL-1β, TNF-α, and RANTES (OptEIA OPTEIA™ ELISA Sets; BD-Pharmingen) were quantified in culture supernatants. For *in vitro* cytokine production, mouse splenocytes (pre-stimulated with 5 µg/ml ConA; Sigma for 16h, and then purified as described above) or synovial cells were incubated with an anti-mouse CD69, clone CD69.2.2 (mouse IgG1), or control mouse IgG1 mAb (both at 10 µg/ml) plus a goat anti-mouse IgG, Fc fragment specific (F(ab')<sub>2</sub>, Jackson Immunoresearch, West Grove, Pennsylvania) at 20 µg/ml. Then, active and total TGF-β1, IL-1β, TNF-α, and RANTES were assayed in culture supernatants after 24 h, as above. In addition, human synovial fluid leukocytes from patients with inflammatory joint

diseases (RA, reactive arthritis, ankylosing spondylitis) were treated at  $1 \times 10^6$  cells/ml with the anti-human CD69 TP1/8 or with an isotype control mAb, with or without cross-linking. TGF- $\beta$ 1 was assayed in culture supernatant after 24 h, as above.

Please amend the paragraph beginning on page 116, line 28 of the instant specification as follows.

**TGF- $\beta$  and MCP-1 production and ELISA in anti-tumor response.** For *in vitro* TGF- $\beta$  production, purified CD3 $^{+}$  T cells from single cell suspensions of lymph nodes and spleen of C57BL/6 or BALB/c mice were obtained. For isolation of CD3 $^{+}$  T lymphocytes, cells were incubated with anti-MAC-1 mAb, followed by two panning steps on petri dishes coated with a rabbit anti-mouse/rat IgG (DAKO, Denmark) at 4°C. The average purity of CD3 $^{+}$  cells was >95%, as determined by flow cytometry analysis. T lymphocytes were stimulated ( $2 \times 10^6$  cells/ml) with plate-bound anti-CD3 mAb (1  $\mu$ g/ml). Cells were added in 200  $\mu$ l of serum-free Stem Span (Stem Cell Technologies Inc., Vancouver, BC) medium in flat-bottomed 96-well plates. Purified anti-CD69, clone CD69.2.2 (IgG1- $\kappa$ ), or control mouse IgG1 mAb were added at 20  $\mu$ g/ml. For cross-linking, F(ab') $_2$  fragments of goat anti-mouse IgG F(ab') $_2$  Fc specific (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were added at a final concentration of 20  $\mu$ g/ml. PD98059 (Calbiochem, San Diego, CA) was added 1h before addition of mAb and the final concentration of PD98059 was 20  $\mu$ M. Cultures were incubated (37 °C, 5% CO<sub>2</sub>), supernatants were collected after 72 h, and secreted TGF- $\beta$  assayed using a TGF- $\beta$ 1 Emax EMAX® ImmunoAssay System (Promega Corp., Madison, WI) ELISA kit, according to the manufacturer's protocol. To measure MCP-1 production, 1 ml of thioglycollate solution (3% w/v; Sigma, St. Louis, MO) was injected i.p. into CD69 $^{-/-}$  and wt mice. Mice were euthanized 3 days later, and leukocyte infiltrate was recovered by peritoneal lavage using 5 ml cold RPMI 1640 with 2% FCS. Peritoneal cells were added in 1 ml of complete medium in flat-bottomed 24-well plates and stimulated overnight with LPS (1  $\mu$ g/ml) at  $2 \times 10^6$  cells/ml. Culture supernatants were collected and MCP-1 was assayed using a mouse MCP-1 OptEIA OPTEIA™ ELISA Set (BD PharMingen).

Please amend the paragraph beginning at page 118, line 29 of the instant specification as follows.

**IL-2 activated NK cells.** Purified spleen NK cells were obtained using the CELLection CELLECTION™ Biotin Binder Kit (Dynal, Oslo, Norway) and biotinylated anti-DX5 antibody according to manufacturer's instructions. Briefly, viable single cell suspensions were incubated (1 h at 37°C ) on polystyrene tissue culture dishes (Becton Dickinson, Mountain View, CA). Nonadherent spleen cells were incubated (15 min, 4°C) with biotinylated anti-DX5 antibody, washed twice, and CELLection CELLECTION™ biotin magnetic Dynabeads DYNABEADS® ( $1 \times 10^7$  magnetic beads per  $1 \times 10^6$  cells) added to capture antibody-coated cells. Cell purity was always >95 % DX5<sup>+</sup>. NK cells were cultured in complete medium with 20 % inactivated FCS, alone or with 1000 UI/ml human rIL-2 (72 h).

Please amend the paragraph beginning at page 119, line 8 of the instant specification as follows.

**<sup>51</sup>Cr-release assays.** Direct NK cell cytotoxic activity was assessed by a standard <sup>51</sup>Cr release assay. In all experiments,  $5 \times 10^3$  Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub>-labeled YAC-1 target cells were mixed with effector cells at the ratios indicated (4h, 37°C). Spontaneous <sup>51</sup>Cr release was determined by incubating target cells with medium alone; maximum release was determined by adding Triton TRITON X100® at a final concentration of 2.5 %. The percentage of specific lysis was calculated as % specific lysis = [(sample cpm – spontaneous cpm) / (maximal cpm – spontaneous cpm)] x 100. Spontaneous <sup>51</sup>Cr release was always <10%, and all experiments were performed in triplicate.

Please amend the paragraph beginning on page 119, line 17 of the instant specification as follows.

**Cell death assays.** Splenocytes ( $4 \times 10^6$  cells/ml) from challenge mice (3 days with  $10^5$  RM-1) were cultured in 24-well plates (Costar, Cambridge, MA) and cell death was assayed at 24 hours after the initiation of culture. Cell Cycle was monitored, cells were stained with propidium iodide (PI), and apoptosis was determined by flow cytometric analysis on an XL™ cytometer (Beckman Coulter, Fullertone, CA). Data are expressed as mean ± SE (n=9). Caspase-3 activity was assayed at 48 hours after initiation of culture by incubation with the PhiPhiLux-G1D2 substrate solution (OncolImmunin, College Park, MD) according to the manufacturer's protocol. Flow cytometric analysis was performed within 60 min of the end of the incubation

period using an XL<sup>TM</sup> cytometer. Peritoneal cells ( $1 \times 10^6$ ) from untreated mice were cultured in 24-well plates and cell death was assessed, at indicated time points, by PI staining. PI<sup>+</sup>-stained samples were considered apoptotic cells.

Please amend the paragraph beginning on page 119, line 30 of the instant specification as follows.

**Western Blot Analysis.** C57BL/6 T cells were preactivated with plate-bound anti-CD3 (5 µg/ml) in serum free medium (37°C, overnight). Cells were harvested, washed twice, and then rested for 4h in serum free medium. Preactivated T cells were incubated in medium with anti-CD69 (IgG1) or control mouse IgG1 mAb (each at 10 µg/ml) for 30 min on ice. Cells were washed twice with cold medium and goat anti-mouse IgG F(ab')<sub>2</sub> Fc specific (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added at 20 µg/ml in 37°C medium. At the time indicated, the reaction was terminated by addition of ice-cold PBS, after which the cells were centrifuged. Pelleted cells were lysed in ice-cold lysis buffer (62.5 mM Tris-HCl, pH:6.8 at 25°C, 2 % w/v SDS, 10% glycerol, 50 mM DTT, 0.01 % w/v bromophenol blue) containing protease and phosphatase inhibitors. After centrifugation (13.000 g, 20 min), to remove insoluble cell fragments, cell lysate supernatant was separated on a 12% SDS-PAGE gel, transferred to Immun-Blot IMMUN-BLOT® PVDF membrane (BioRad, Hercules, CA) and blotted with the indicated antibody. Polyclonal anti-phospho-Erk 1/2 antibody was from Calbiochem (San Diego, CA). The blot was stripped and reprobed with Erk kinase (Erk 1/2) monoclonal antibody (Zymed Lab. Inc. San Francisco, CA) to confirm equal protein loading. Western blotting was performed using anti-rabbit-HRP or anti-mouse-HRP and visualized by enhanced chemiluminescence.